Biochemical Pharmacology, Vol. 16, pp. 1719–1728. Pergamon Press Ltd. 1967. Printed in Great Britain

LOCALIZATION OF DIFFERENT STEPS IN NORADRENALINE SYNTHESIS TO DIFFERENT FRACTIONS OF A BOVINE SPLENIC NERVE HOMOGENATE

L. STJÄRNE and F. LISHAJKO

Department of Physiology, Karolinska Institutet, Stockholm, Sweden

(*Received* 14 *December* 1966; *accepted* 31 *January* 1967)

Abstract—Experiments were carried out to determine to what extent the synthesis of noradrenaline in bovine splenic nerve tissue can proceed in the high-speed sedimentable particulate fraction that contains the specific noradrenaline storage vesicles. Various fractions of a homogenate of this tissue were incubated in a potassium phosphate medium together with radioactively labeled precursors of noradrenaline. The first two steps in the synthesis, the ring hydroxylation of tyrosine and the decarboxylation of dopa, were shown not to require the presence of this particulate fraction. On the other hand, this fraction was an absolute prerequisite for the last step, the β-hydroxylation of dopamine. No significant formation of catechol compounds from tyrosine could be demonstrated on incubation with this particulate fraction after it had been washed and resuspended in potassium phosphate. Some dopa decarboxylase activity remained in the particulate fraction even after washing. The results support the view that in this tissue only one enzyme involved in noradrenaline synthesis, dopamine β-hydroxylase, is truly located in the above-mentioned particles, possibly inside the membrane of the noradrenaline-storing vesicles. Tyrosine hydroxylase and dopa decarboxylase appear to be located outside these particles. Dopa decarboxylase shows some association with the particulate fraction studied, and might be, to some extent, bound to the outer aspect of the same particles which contain the β-hydroxylase, or could just be accumulated in the perivesicular parts of the probably highly organized axoplasm.

THE DEMONSTRATION by Goodall and Kirshner[1] of formation of noradrenaline (NA)* from tyrosine (T) in homogenates of sympathetic nerve tissue provided final evidence that the nerve trunk and terminals can manufacture their own neurotransmitter from its dietary precursor, tyrosine. Their results also supported the hypothesis originally proposed by Blaschko[2] and Holtz[3] that the major biosynthetic pathway for the formation of catecholamines involves the sequence T–dihydroxyphenylalanine (dopa)–dopamine (DA)–NA.

Until recently, little experimental work had been done to clarify the subcellular localization of these three steps in the biosynthesis of NA in sympathetic nerve or chromaffin tissue. The earliest evidence obtained concerned the intermediate step, the decarboxylation of dopa, which was reported to proceed in the particle-free cytoplasm of adrenal medullary cells.[4] By analogy, it has been generally assumed that this process, in nerve tissue, takes place in the axoplasm. The last step, the

* Abbreviations: T = tyrosine, dopa = dihydroxphenylalanine, DA = dopamine, NA = noradrenaline.

β-hydroxylation of DA, has been demonstrated to proceed in the specific catecholamine-storing particles of the adrenal medullary cells.[5] The finding of DA β-hydroxylase activity in the NA-containing particulate fraction isolated from the rat heart[6] suggests that this step in NA synthesis in noradrenergic nerves also may take place in specific NA storage vesicles. The enzyme catalyzing the first step, the hydroxylation of tyrosine, has recently been claimed to be particle-bound.[7,8] Moreover, the whole of the biosynthesis of NA from tyrosine was reported to proceed in this particulate fraction, in adrenal medulla, brain, and various sympathetically innervated organs.[7–9]

In the present experiments bovine splenic nerve tissue was used for a study of the subcellular localization of the different steps in NA biosynthesis *in vitro*. Various fractions of the nerve homogenate were incubated with radioactively labeled precursors of NA in order to determine to what extent the above-mentioned biosynthetic sequence could proceed in the particle-free supernatant obtained by high-speed centrifugation of the nerve homogenate, and to what extent the particulate fraction was required for the complete NA synthesis from tyrosine.

MATERIALS AND METHODS

Fresh bovine splenic nerve tissue was obtained from the slaughterhouse within 30 min after the animals were killed and was immediately chilled with ice. All subsequent steps, except the incubation, were carried out at 0–5°.

After carefully removing contaminating tissue and desheathing the nerves, they were homogenized in an Ultra-Turrax apparatus. In some experiments the nerve tissue was instead squeezed between nylon rollers. The whole-nerve homogenate, or the pressed juice, was suspended in ice-cold isotonic potassium phosphate, pH 7·5 about 1 g nerve tissue/8 ml (preparation 1), and centrifuged at 9000*g* for 10 min The supernatant from this centrifugation, which contained the different species of high-speed sedimentable particles (HS particles), including the specific NA-storing vesicles, suspended in a potassium phosphate dilution of their original cytoplasmic medium (preparation 2), was centrifuged at 50,000*g* for 30 min (or in some experiments at 100,000*g* for 40 min). The particle-free high-speed supernatant (preparation 3) was used for some incubations. The high-speed sediment was resuspended in potassium phosphate (preparation 4, resuspended HS particles) and recentrifuged at 50,000*g* for 30 min. This sediment was resuspended a second time in potassium phosphate (preparation 5, washed and resuspended HS particles).

Incubations

Eight-ml volumes of the different preparations were incubated in stoppered plastic centrifuge tubes at 20° for 20–60 min in the presence of various fortifiers and substrates.

Hydroxylation of tyrosine. Preparations 1–5 were incubated in the presence of tritium-labeled tyrosine (New England Nuclear Corp., l-tyrosine-3,5-^{3}H, sp. act. 4 C/m-mole) or, in some experiments, with ^{14}C-labeled tyrosine (New England Nuclear Corp., L-tyrosine-^{14}C, uniformly labeled, sp. act. 300 mc/m-mole), 1 μc/ml, with or without fortification by tetrahydrofolate 5 mM, $FeSO_4$ 0·5 mM, mercaptoethanol 100 mM, ATP 3 mM, and $MgSO_4$ 3 mM. The endogenous tyrosine content of the high-speed sediment and of the particle-free supernatant (preparation 3) was determined in some experiments by the fluorimetric method of Waalkes and Udenfriend.[10]

Decarboxylation of dopa. Preparations 2–5 were incubated in the presence of ^{14}C-labeled dopa (New England Nuclear Corp., DL-3, 4-dihydroxyphenylalanine-2-^{14}C, sp. act. 2·6 mC/m-mole, 1 μC/ml, with or without pyridoxal phosphate 0·4 mM, ATP 3 mM, and $MgSO_4$ 3mM as fortifiers.

Hydroxylation of DA. Preparations 2–4 were incubated with tritium-labeled DA (New England Nuclear Corps., 3, 4-dihydroxyphenylethyl-1-^{3}H-amine-HBr, sp. act. 50 mC/m-mole), 1 μC/ml, in the presence of ATP 3 mM and $MgSO_4$ 3mM.

Analysis

The incubated high-speed supernatant and, in some experiments, the whole of the incubated particle suspension, were extracted with 0·8 ml of 4 M perchloric acid. In other experiments the particles were separated from the suspension medium prior to extraction. In these cases the suspension was centrifuged at 50,000*g* for 30 min, the supernatant was decanted, and the sediment was resuspended in 9 ml potassium phosphate and centrifuged as described above. The supernatant and wash were extracted with 0·8 ml of 4 M perchloric acid, and the washed sediment with 0·5 ml of 0·4 M perchloric acid, after which it was diluted with 3·5 ml of glass-distilled water. Unlabeled NA, DA, dopa or tyrosine, 50 μg of each was added to the extracts, and the protein precipitate was removed by centrifugation. In the tyrosine experiments the extracts were passed through an alumina column, at pH 8·2, allowing the tyrosine to pass quantitatively into the effluent and water wash, while dopa and amines were retained. These were then eluted with two 3-ml portions of 0·2 M perchloric acid. After bringing the eluates, as well as the extracts not passed through alumina, to pH 4–5 with potassiun hydroxide and removing the perchlorate precipitate by centrifugation, the extracts were analyzed by cation-exchange column chromatography on Amberlite CG 120, type 2 (200–400 mesh). The column size was 4 × 100–120 mm. The resin was washed with 1·0 N sodium acetate, pH 6·5, and then rinsed with glass-distilled water. After the extracts were adsorbed to the resin and the acid metabolites were washed out with 10–20 ml of water, dopa was eluted with 10–20 ml of 1·0 N sodium acetate, pH 4·0. In most experiments gradient elution was used to separate the amine. The reservoir was filled with 2N hydrochloric acid, and the mixer (25 ml) with water. Flow rate was adjusted to about 4 ml/hr. The effluent was collected in fractions of about 2 ml by means of an automatic fraction collector. The positions of the carrier NA, DA, and dopa were determined by reading the spontaneous fluorescence at 335 mμ (activation wavelength 285 mμ) in an Aminco-Bowman spectrofluorophotometer. The radioactivity of the different fractions was measured by counting 0·5-ml aliquots of the eluate in a 7:3 toluene:absolute ethanol solution containing 4 g of 2·5-diphenyloxazole and 100 mg of 1,4-*bis*-2(4-methyl-5-phenyloxazolyl) benzene per liter of toluene. The counting time was 10 min. Quenching was monitored by internal standards. The overall recovery of material put through the entire procedure ranged between 70 and 90 per cent.

RESULTS

β-Hydroxylation of dopamine. The particulate fraction in the low-speed supernatant was found to be essential for the β-hydroxylation of DA. Thus incubation of the particle-free supernatant (preparation 3) with ^{3}H-DA did not result in noradrenaline formation. This process proceeded both in the HS particle-containing low-speed

supernatant (preparation 2) and in resuspended HS particles (preparation 4), although the yield of NA formed in the latter was lower.

Decarboxylation of dopa. The presence of HS particles was not required for the decarboxylation of dopa. Thus incubation of the high-speed supernatant with labeled dopa led to the formation of DA, but not of NA. On the other hand, NA as well as DA was formed from dopa on incubation of the low-speed supernatant (preparation 2). In the absence of fortification of the medium with pyridoxal phosphate, ATP-Mg^{2+} the catecholamine formation from dopa in resuspended HS particles (preparation 4) was very low (Fig. 1). However, the yield of DA and NA formed in this preparation, after fortification, was almost as high as in the unfortified low-speed

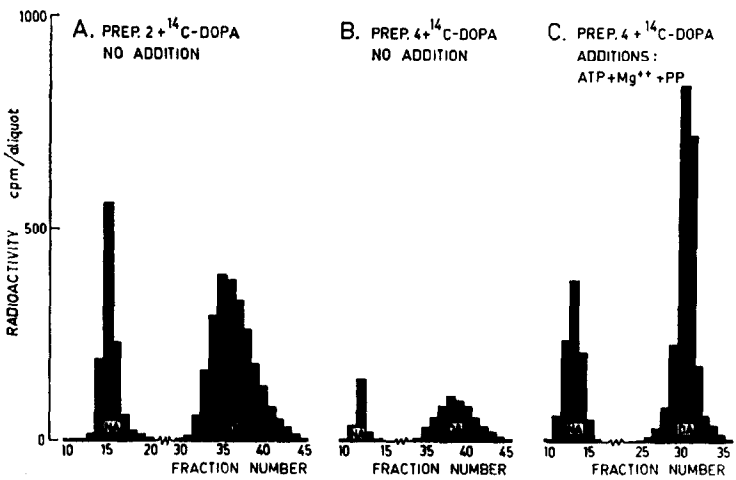

FIG. 1. Ion-exchange chromatograms of extracts of the high-speed sediment pellets after incubation of preparation 2 (low-speed supernatant) or 4 (resuspended HS particles) with ^{14}C-dopa, 1μC/ml, at 20° for 60 min, with or without addition of ATP 3 mM, $MgSO_4$ 3mM, and pyridoxal phosphate (PP) 0·4 mM.

supernatant (preparation 2). On the other hand, the catecholamine formation from dopa in the washed and resuspended HS particles (preparation 5) amounted to but a few per cent of that in experiments with HS particles suspended in their original medium (preparation 2).

Hydroxylation of tyrosine

Turrax preparations + ^{3}H-T. Incubation of the whole-nerve homogenate with tritium-labeled tyrosine, in a medium fortified with tetrahydrofolate, $FeSO_4$, ATP, and Mg^{2+}, consistently led to the formation of DA and NA (Fig. 2). So did similar incubation of the low-speed supernatant (preparation 2) with ^{3}H-T (Fig. 3). About one-half of the NA formed was recovered from the high-speed sediment; the remainder was located in the supernatant. Of the newly formed DA, less than 1/10 was found in the sediment fraction. Omitting tetrahydrofolate from the incubation medium reduced the yield of DA and NA by about one-half.

Incubation of HS particles resuspended in potassium phosphate (preparation 4) with ^{3}H-T also led to some DA and NA formation (Fig. 3). In this case the yield of amines formed was much lower, from 1–15 per cent of that obtained on incubation

of the whole low-speed supernatant. The sedimented pellet from the resuspended HS particle experiments contained one-third to one-half the NA formed, but no detectable amounts of DA.

On incubation of the particle-free supernatant (preparation 3) with ^{3}H-T no NA was formed, but the amount of DA formed was approximately equal to the sum of

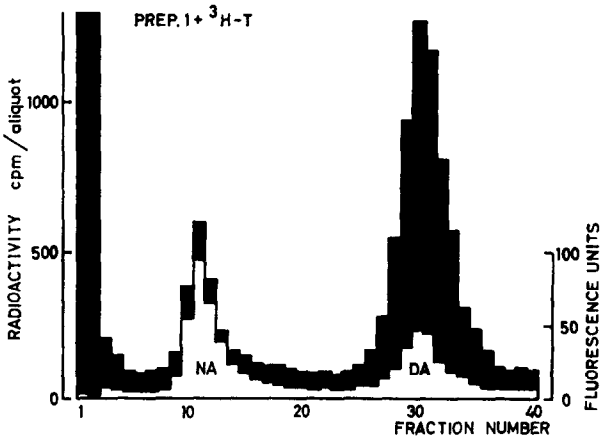

FIG. 2. Ion-exchange chromatogram of extract of whole-nerve homogenate (preparation 1) after incubation with ^{3}H-T, 1 μC/ml, at 20° for 60 min in the presence of tetrahydrofolate 5 mM, ATP 3 mM and $MgSO_4$ 3 mM. Open columns: fluorescence at 335 mμ (activation wavelength 285 mμ (Filled columns: radioactivity.

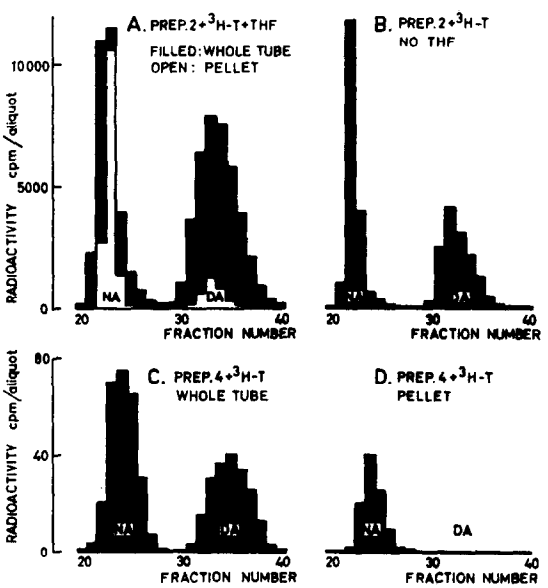

FIG. 3. Ion-exchange chromatograms of extracts of whole tubes or of the high-speed sediment pellets after incubation of preparation 2 (low-speed supernatant) or preparation 4 (resuspended HS particles) with ^{3}H-T, 1 μC/ml, at 20° for 60 min, with or without tetrahydrofolate (THF) 5 mM, ATP 3 mM, and $MgSO_4$ 3 mM.

the DA and NA formed on incubation of the HS particle-containing low-speed supernatant (Fig. 4).

Squeezed preparations + ^{14}C-T or ^{3}H-T. In order to exclude artifacts in the results of the tyrosine experiments, owing to the homogenization method or to isotopic effects, the experiments described above were repeated with the different fractions

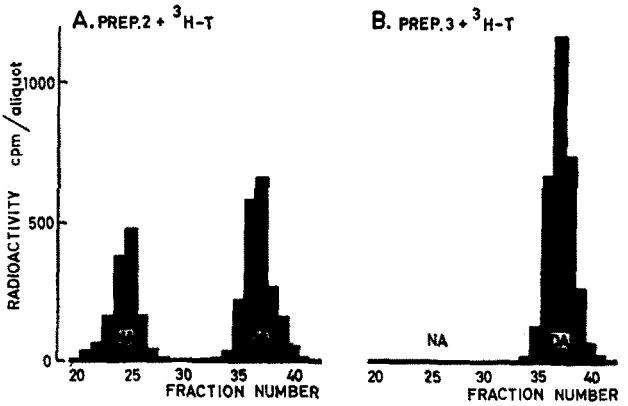

FIG. 4. Ion-exchange chromatograms of extracts of whole tubes after incubation of preparation 2 (low-speed supernatant) or preparation 3 (high-speed supernatant) with ^{3}H-T, 1 μC/ml, at 20° for 60 min in the presence of ATP 3 mM and $MgSO_4$ 3 mM.

of the press juice obtained by squeezing the nerve tissue, and with the use of ^{14}C-labeled tyrosine. In preparations 4 and 5, unlabeled tyrosine was added to a final concentration of unlabeled exogenous tyrosine of 2×10^{-5}M. The incubation medium was fortified with tetrahydrofolate, $FeSO_4$, ATP and Mg^{2+}. Occasional addition of mercaptoethanol, 100 mM, did not improve the yield of catechols formed from tyrosine and was therefore omitted.

The results obtained in this series were qualitatively essentially identical with those found with the Turrax preparation and with the use of ^{3}H-T. The total catechol formation from tyrosine was found to be virtually independent of the presence of HS particles. Thus the amount of catechols formed in the HS particle-containing low-speed supernatant (preparation 2) and in the HS particle-free high-speed supernatant (preparation 3) was identical, and followed the same time course. In the washed and resuspended HS particles (preparation 5), fortified as described above and containing unlabeled exogenous tyrosine, 2×10^{-5}M, no catecholamine formation was demonstrable. However, a small radioactive peak corresponding to dopa was observed in the chromatograms from this preparation also (Fig. 5). The total radioactivity of this peak corresponded to about 6 per cent of that of the sum of the different catechols formed in preparations 2 and 3. Since the total (endogenous + exogenous) tyrosine content of these latter preparations was about $0{\cdot}9 \times 10^{-5}$M, as compared to $2{\cdot}4 \times 10^{-5}$M in preparation 5, this would correspond to an absolute formation of catechol compounds in the washed and resuspended HS particles of

16 per cent (0·11 nanomole) of that in the preparation containing a potassium phosphate dilution of the axoplasm, with or without HS particles (preparations 2 and 3, 0·68 nanomole).

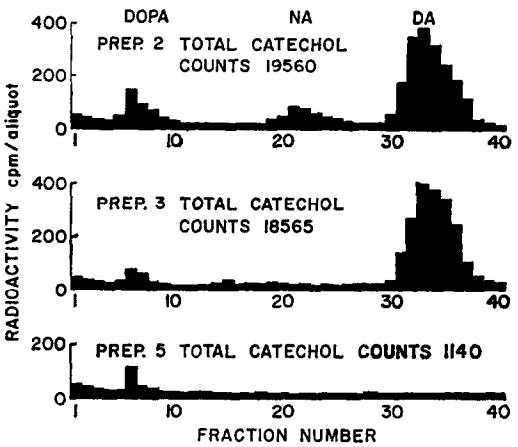

FIG. 5. Ion-exchange chromatograms of extracts of whole tubes after incubation with ^{14}C-T, 1 μC/ml, at 20° for 60 min in the presence of tetrahydrofolate 5 mM, $FeSO_4$ 0·5 mM, ATP 3 mM, $MgSO_4$ 3 mM, and (in c) unlabeled tyrosine 2 × 10^{-5}M. The following preparations from the squeezed nerve tissue were used: (a) low-speed supernatant (preparation 2); (b) high-speed supernatant (preparation 3); (c) washed and resuspended HS particles (preparation 5).

DISCUSSION

In accordance with the original observation of Goodall and Kirshner[1] the bovine splenic nerve homogenate was in the present experiments shown to be able to form noradrenaline from tyrosine. The results obtained provide information concerning the subcellular localization of the three biosynthetic steps.

The HS particles were consistently found to contain all the DA β-hydroxylase activity in this tissue. For several reasons it appears likely that this enzyme is located in specific NA-storing vesicles or in particles with similar properties. This is supported by previous evidence of DA β-hydroxylase activity in the catecholamine-storing vesicles of the adrenal medulla[5] and in the NA-containing particulate fraction isolated from the rat heart.[6] It is also indicated by the finding in this laboratory that the formation of NA from DA can be inhibited by drugs like reserpine,[11] known to block catecholamine uptake into the specific NA-containing vesicles, [12] or by high concentrations of NA in the medium.[13] These observations suggest that the DA β-hydroxylation step occurs only after DA has been taken up into specific NA-storing vesicles, and that therefore the enzyme involved is located inside the membrane of these vesicles.[5, 14, 15]

Although no β-hydroxylation of DA could be demonstrated except in the presence of HS particles, the ring hydroxylation of tyrosine or the decarboxylation of dopa was found to be independent of these particles, proceeding to the same extent and with the same time course in the particle-free high-speed supernatant as in the HS particle-containing low-speed supernatant. On the other hand, not even washing and resuspension could completely remove dopa decarboxylase activity from the HS particles.

These findings demonstate differences in the degree of association with the HS particles among the three enzymes involved in NA biosynthesis. Only DA β-hydroxylase occurs exclusively in this particulate fraction, whereas at least the major portion of the tyrosine hydroxylase and dopa decarboxylase, according to the present results, is located outside these particles. It appears very unlikely that this is due to almost quantitative leakage of originally HS particle-bound enzymes into the medium on homogenization of the tissue, since almost all the tyrosine hydroxylase and dopa decarboxylase activity appeared in the particle-free supernatant even when the tissue was homogenized by squeezing between nylon rollers at 0°, a procedure that is not likely to disrupt the membranes of the HS particles.

The low-degree tyrosine hydroxylase and dopa decarboxylase activity present in the resuspended HS particle preparation (preparation 4) could be further reduced by washing (preparation 5). The small chromatographic peak corresponding to dopa, found in the washed and resuspended particles incubated with tyrosine in the presence of tetrahydrofolate, $FeSO_4$, ATP, and Mg^{2+}, could not be positively identified. Moreover, no DA or NA formation from tyrosine could be demonstrated in this preparation. Thus there is little evidence that tyrosine hydroxylation proceeds in the same particles which β-hydroxylate DA.

Dopa decarboxylase activity could not be completely washed out of the HS particles, indicating that decarboxylation of dopa might to some extent proceed in the HS particles. [16, 17] Thus when the resuspended HS particle preparation (preparation 4) was incubated with dopa, fortification of the medium with pyridoxal phosphate and ATP–Mg^{2+} increased the otherwise low yield of DA and NA formed from dopa almost to the level obtained in the original unfortified low-speed supernatant (preparation 2). This observation might suggest that the resuspended HS particles did not lack the enzyme, dopa decarboxylase, but rather the cofactors, pyridoxal phosphate and ATP–Mg^{2+}. However, further washing strongly reduced the dopa decarboxylase activity in HS particles. Moreover, the amount of newly formed catecholamines ultimately recovered from the washed high-speed sediment at the end of the incubation period is not only a function of absolute synthesis but also of the spontaneous loss of catecholamines from the HS particles during the incubation period, which is strongly counteracted by ATP–Mg^{2+}. Thus the results do not provide evidence for any quantitatively important dopa decarboxylase activity in the HS particles.

The evidence in the literature for the concept that the whole of the synthesis of NA proceeds in one species of intracellular organelles does not appear to be conclusive. It is based on experiments with fractions obtained from homogenates of adrenal medulla, brain, and various peripheral sympathetically innervated organs.[7–9] However, the reports from these experiments do not explicitly state that the coarse tissue particles, including intact or partially disrupted cells, were removed from the homogenate by a preliminary low-speed centrifugation. Moreover, the particulate fraction from brain homogenates shown to contain the tyrosine hydroxylase activity was defined electronmicroscopically as consisting essentially of synaptosomes.[8] Thus the particulate fractions studied appear to have contained both cell membranes, cytoplasm, and intracellular organelles. The presence of enzymatic activity in these particulate fractions does not seem to provide any information about the subcellular distribution of the enzyme. Moreover, the finding that the major part of the tyrosine hydroxylase activity sedimented in 0·32 M sucrose at 15,000–20,000*g* as well as the

absence of such enzymatic activity in the sediment on subsequent centrifugation at 105,000*g* in fact excludes the possibility that the enzyme could be located in the 'microsomal fraction' in which the specific NA-storing vesicles are found,[6] and rather suggests that it might be contained in heavier intracellular organelles.[9, 16] The observation that a considerable proportion of the tyrosine hydroxylase activity in these experiments was recovered from the high-speed supernatant, which was interpreted as leakage of particle-bound enzyme into the medium, could also support the opposite view, that the tyrosine hydroxylase activity found in the supernatant in fact represented enzyme physiologically located in the cytoplasm, whereas the activity observed in the heterogeneous 15,000–20,000*g* sediment could be due to contamination of intracellular organelles with cytoplasmic material because of incomplete homogenization.

Thus the accumulated evidence, both from the literature and from the present experiments, appears to suggest that the enzymatic apparatus for the first two steps in NA biosynthesis is at least much less intimately related to the HS particles than that for the last step. It might be argued that this *in vitro* difference could be artifactual and represent differences in the degree of leakage of originally particle-bound enzymes on disruption of the tissue. However, according to the present results, DA β-hydroxylase, and only that enzyme, was found exclusively in the HS particles, probably inside the membrane of the NA-storing vesicles. It appears unlikely that protein molecules such as those of tyrosine hydroxylase and dopa decarboxylase, which were almost quantitatively recovered from the particle-free supernatant, could be located inside the membrane of the vesicles *in vivo* but leak out so completely during homogenization at 0°. Thus it appears quite safe to conclude that the present findings reflect the physiological distribution of the enzymes, and that the first two steps in NA synthesis proceed outside, or on the exterior aspect of the membrane of the NA-storing vesicles, while the last steps take place inside this membrane. This concept is also supported by the observation above that more than half the NA, but less than a tenth of the DA, formed from tyrosine in the low-speed supernatant (preparation 2) was recovered from the washed high-speed sediment, while the remainder was found in the supernatant. Furthermore, drugs known to specifically block catecholamine uptake into the NA-storing vesicles, such as reserpine, inhibited exclusively NA formation from DA, without affecting DA formation from tyrosine.[11, 15]

Experiments are in progress to analyze further this issue by kinetic studies of the different steps in NA synthesis in the bovine splenic nerve preparation.

Acknowledgement—This investigation was supported by grants from the Swedish Medical Research Council, projects No. 14 X–97–02 and 14 X–625–01, which are hereby gratefully acknowledged.

REFERENCES

1. McC. Goodall and N. Kirshner, *Circulation Res.* **17**, 366 (1958).
2. H. Blaschko, *J. Physiol., Lond.* **96**, 50P (1939).
3. P. Holtz, *Naturwissenschaften* **27**, 724 (1939).
4. H. Blaschko, P. Hagen and A. D. Welch, *J. Physiol., Lond.* **129**, 27 (1955).
5. N. Kirshner, *J. biol. Chem.* **237**, 2311 (1962).
6. L. T. Potter and J. Axelrod, *J. Pharmac. exp. Ther.* **142**, 299 (1963).
7. T. Nagatsu, M. Levitt and S. Udenfriend, *J. biol. Chem.* **239**, 2910 (1964).
8. P. L. McGeer, S. P. Bagchi and E. G. McGeer, *Life Sci.* **4**, 1859 (1965).
9. S. Udenfriend, *Harvey Lect.* Series 60, p. 57. Academic Press, New York (1964–65).

10. T. P. WAALKES and S. UDENFRIEND, *J. Lab. clin. Med.* **50**, 733 (1957).
11. L. STJÄRNE and F. LISHAJKO, *Br. J. Pharmac.* **27**, 398 (1966).
12. U. S. VON EULER and F. LISHAJKO, *Int. J. Neuropharmac.* **2**, 127 (1963).
13. L. STJÄRNE, R. H. ROTH and F. LISHAJKO, *Nature, Lond.* in press.
14. L. STJÄRNE, R. H. ROTH and F. LISHAJKO, *Biochem. Pharmac.* **16**, 1728 (1967).
15. L. STJÄRNE, *Acta physiol. scand.* **62**, 441 (1966).
16. L. T. POTTER, *Pharmac. Rev.* **18**, 439 (1966).
17. L. STJÄRNE, *Pharmac. Rev.* **18**, 425 (1966).